Docket No. 96700/1023

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

| | | |
|---|---|---|
| Applicants | : | Seetharama A. Acharya and Belur N. Manjula |
| Appl. No. | : | 10/538,976 |
| Filing Date | : | December 5, 2005 |
| Title | : | MODIFIED HEMOGLOBIN AND METHODS OF MAKING SAME |
| Art Unit | : | 1656 |
| Examiner | : | Samuel W. Liu, Ph.D. |
| Customer No. | : | 1912 |

**Declaration under 37 C.F.R. §1.131 of Seetharama A. Acharya and Belur N. Manjula**

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

We, Seetharama A. Acharya and Belur N. Manjula, hereby declare as follows:

1. We are each a co-inventor of the subject matter claimed in U.S. Patent Application No. 10/538,976.

2. Seetharama A. Acharya, Ph.D., is currently a Professor of Medicine (Hematology) and Professor of Physiology & Biophysics at Albert Einstein College of Medicine, Bronx, New York.

   At the time of the invention, Belur N. Manjula, Ph.D. was a Senior Associate in the Department of Physiology & Biophysics at Albert Einstein College of Medicine, Bronx, New York. Belur N. Manjula is currently retired.

3. We understand that the broadest pending claim of the application is directed to a process for preparing a modified hemoglobin molecule (Hb), comprising

the steps of: (a) reacting Hb with 8-15 fold excess of iminothiolane to form thiolated Hb; and (b) reacting the thiolated Hb with 16-30 fold excess of PEG functionalized with a maleimide moiety, to form the modified Hb.

4. We understand that the claims are currently rejected as being anticipated by Winslow et al. (U.S. Patent No. 6,974,795 B2), which has a priority date of January 11, 2002.

5. We attach a redacted page from a laboratory notebook of experiments performed under our direction in our laboratory at Albert Einstein College of Medicine, Bronx, New York, prior to the January 11, 2002 priority date of Winslow et al. The notebook page is dated prior to January 11, 2002; the date has been redacted on the attached copy. The notebook page describes the preparation of HbA-(PEG-5000)$_6$ pegylated hemoglobin by reacting HbA with a 10 fold excess of Traut's reagent (2-iminothiolane) and reacting the thiolated HbA with 20 fold excess PEG-5000.

6. We hereby declare that all statements made herein and of our knowledge are true and that all statements made on information and belief are believed to be true; and we further declare that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

A S Acharya

Seetharama A. Acharya Date Nov 16, 2007

B.N. Manjula

Belur N. Manjula Date Nov, 16, 2007

(6)

# Preparation of HbA-(PEG-5000)$_6$

HbA + T.R (1:10)
↓ 4 hrs on ice
+ 20 fold PEG-5000 **REDACTED**
↓
2 hrs on ice.
↓
G-25

Starting material:
HbA : oxy HbA - Ch-300
Traut's reagent
& Mal-PEG-5000 → Biaffinity Systems.

## Thiolation (1:10)

| | Stock | 8.6 ml |
|---|---|---|
| 1 mM HbA (PBS) | 1.44 mM | 6 ml |
| 10 mM T.R (PBS) | 50 mM | 1.72 ml |
| PBS | | 0.88 ml. |

50 mM T.R = 6.88 mgs/ml (16 mgs/23 ml).
M.wt = 137.

↓ Incubated on ice for 4 hrs

PEGulation. (1:20) molar ratio over protein.

## PEGULATION (1:20)

| | Stock | 8.6 ml (8) |
|---|---|---|
| 1 mM thiolated oxy HbA (PBS) | 1 mM | 8.6 ml |
| 20 mM PEG-5000 (PBS) | 100 mM | 1.72 ml |

100 mM PEG-5000 = 500 mgs/ml (990 mgs/1.98 ml PBS)
M.wt ≃ 5000

↓

Incubated further for 2 hrs on ice and gel filtered on G-25 (2.5 × 45) cms using PBS. Eluted sample was concentrated.

↓

Purified on FPLC.